Transgenic TNF-α Causes Apoptosis in Epidermal Keratinocytes After Subcutaneous Injection of TNF-α DNA Plasmid

To the Editor:

Apoptosis is known as programmed cell death and has been recognized as an active regulator in the process of cell proliferation and differentiation. Some activators such as hormones p53 and UVB, can induce apoptosis *in vitro* and *in vivo* (McCall and Cohen, 1991; Gniadecki *et al*, 1997). Tumor necrosis factor-α (TNF-α) is an inflammatory cytokine, which is generally thought to have an antitumor effect. It has been demonstrated that TNF-α can also induce apoptosis in hepatocytes (Senaldi *et al*, 1998), human keratinocytes (Reinartz *et al*, 1996), and normal cytotrophoblasts (Garcia *et al*, 1996) *in vitro*; however, there is a contrary report in which TNF-α is shown not to induce apoptosis in cultured human keratinocytes (Benassi *et al*, 1997), and no report has ever demonstrated induction of apoptosis in TNF-α transgenic mice (Probert *et al*, 1995; Crew *et al*, 1998; Nakama *et al*, 1998). We have previously reported that the human interleukin-10 (Meng *et al*, 1998) and the human interleukin-6 (Sawamura *et al*, 1998) gene can be introduced into epidermal keratinocytes *in vivo* using the plasmid DNA injection method. Here, we have examined the biologic effect of transgenic TNF-α by introducing the human TNF-α gene into epidermal keratinocytes using this method.

First, we constructed a human TNF-α expression plasmid (phTNF-α). The coding region of human TNF-α cDNA was amplified using polymerase chain reaction (PCR) based on its cDNA sequence (Wang *et al*, 1985). For subcloning, primers contained restriction enzyme sites at the 5′-ends (F: 5′-AACT-CGAGATGAGCACTGAAAGCATGATCCGG-3′, R: 5′-AAG-CGGCCGCTCACAGGGCAATGATCCCAAAG-3′). Subcloning was performed using a pCY4B expression vector (Meng *et al*, 1998). Plasmid containing the TNF-α cDNA without any eukaryotic promoter, named phTNF-α(–), was used as a negative control. Plasmid phTNF-α was intradermally injected into the dorsal skin of Hirosaki Hairless Rat (HHR) at a concentration of 0.2 μg per μl (Meng *et al*, 1998). To determine whether keratinocytes could express human TNF-α mRNA after introduction of phTNF-α, reverse-transcriptase (RT)-PCR was performed using RNA samples derived from keratinocytes in the treated area as previously described (Meng *et al*, 1998). PCR products were electrophoresed by a 2% agarose gel. Transgenic TNF-α in local keratinocytes was detected by ELISA (BioSource International, Camarillo, CA) and was expressed as nanograms per microgram of protein (Protein Assay Kit; Bio-Rad, Hercules, CA). Control samples were taken from the pTNF-α(–)-treated skin. Immunohistochemical staining for TNF-α was done using a polyclonal rabbit antihuman TNF-α antibody (Genzyme). Skin samples were taken 24 h after phTNF-α injection and frozen sections were used for the staining. To examine the biologic effect of transgenic TNF-α in local skin, skin specimens were fixed, paraffin-embedded, cut in 5 mm sections and stained with hematoxylin and eosin for routine histologic examination. To detect apoptotic cells in the epidermis, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) technique was performed using an apoptosis detection kit (ApopTag, Oncor, Gaitheerburg, MD).

Our results of RT-PCR showed that a 741 bp band was present in the samples from phTNF-α-transferred keratinocytes, whereas no band was observed in samples from phTNF-α(–)-transferred keratinocytes (**Fig 1*a***). PCR with mRNA samples without a RT step were also performed and no TNF-α DNA band was detected (data not shown). Results of ELISA showed that injection of phTNF-α caused a significant increase of transgenic hTNF-α in

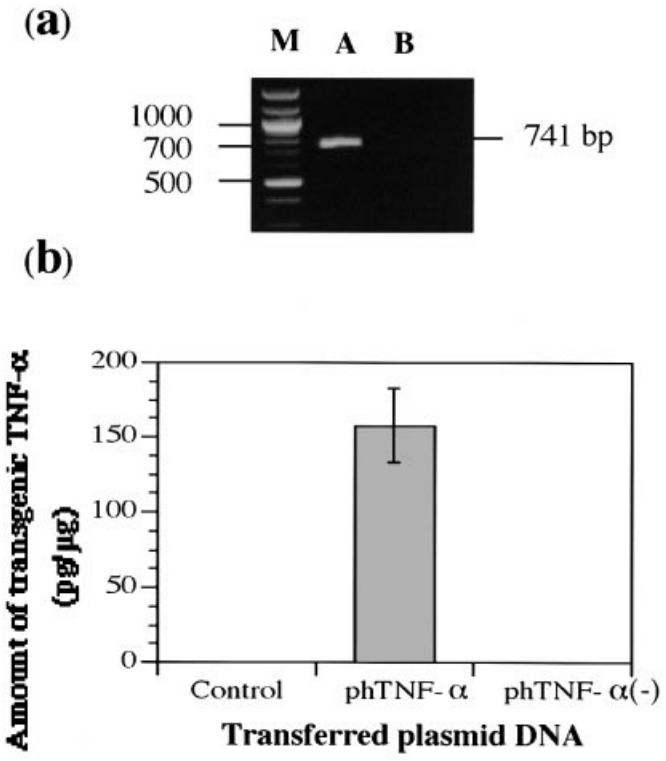

Figure 1. Detection of human TNF-α mRNA expression and transgenic TNF-α in rat keratinocytes after TNF-α gene transfer. (*a*) Total RNA was extracted from rat keratinocytes from the gene-transferred area 24 h after phTNF-α or phTNF-α(–) injection. RT-PCR was performed using primers to amplify TNF-α cDNA. A, phTNF-α injection; B, phTNF-α(–) injection; M, size marker. (*b*) Keratinocytes were obtained from the gene-transferred area 24 h after phTNF-α or phTNF-α(–) injection. Transgenic TNF-α levels were assayed by ELISA. Each value shown represents the mean ± SD of six individual samples.

Manuscript received March 19, 1999; revised June 18, 1999; accepted for publication July 12, 1999.

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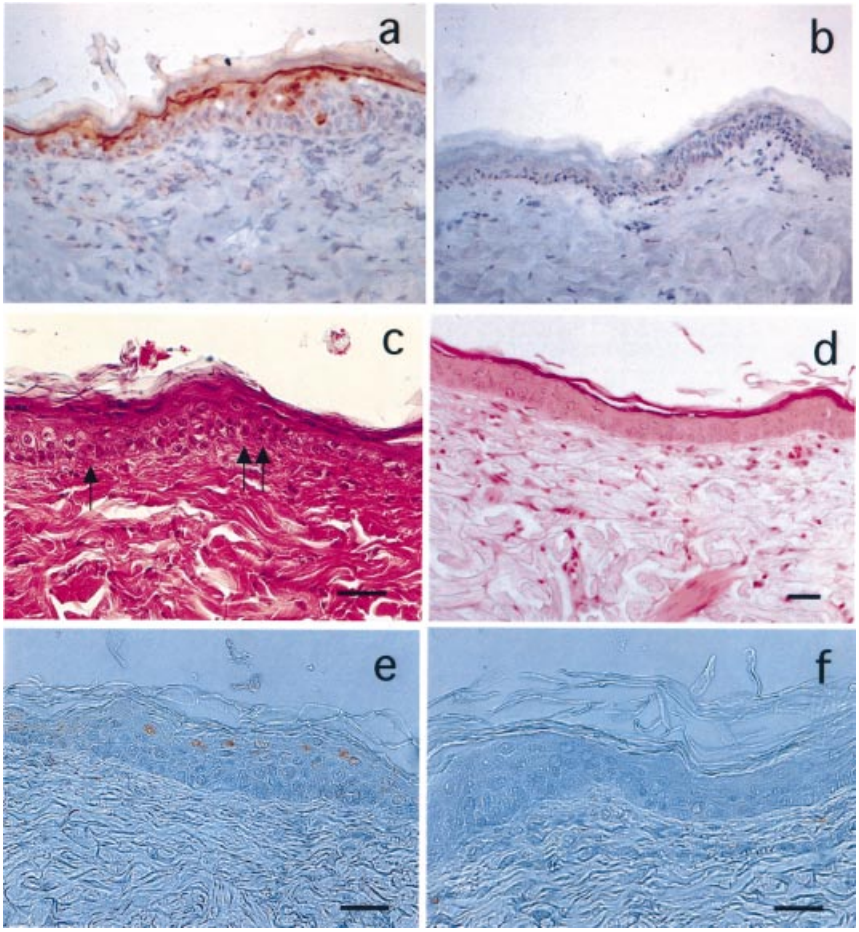

Figure 2. Immunohistochemical staining for transgenic TNF-α and induction of apoptosis by transgenic TNF-α in local skin. TNF-α positive staining of keratinocytes was observed in phTNF-α treated skin (*a*), whereas a negative result was shown in phTNF-α(–) injected skin (*b*). Hematoxylin and eosin staining showed that some keratinocytes appeared eosinophilic in the epidermis (*c*). TUNEL staining showed TUNEL-positive cells in the epidermis, suggesting that transgenic TNF-α induced apoptosis in keratinocytes (*e*). Samples from phTNF-α(–) treated skin showed negative results both in the routine (*d*) and in the TUNEL staining (*f*). *Scale bar*: 25 mm.

local keratinocytes (**Fig 1*b***). Immunohistochemical staining gave a positive staining of keratinocytes in phTNF-α injected skin (**Fig 2*a***), whereas a negative result was shown in phTNF-α(–) injected skin (**Fig 2*b***). Routine staining showed that some keratinocytes appeared eosinophilic in the epidermis (**Fig 2*c***). TUNEL-positive cells were observed in the epidermis, indicating that transgenic TNF-α induced apoptosis in keratinocytes (**Fig 2*e***). Samples from phTNF-α(–) treated skin show negative results in both the routine stain and the TUNEL staining (**Fig 2*d*, *f***).

In this study, human TNF-α gene was introduced into rat skin *in vivo* by injection of its DNA plasmid. The transferred hTNF-α gene was expressed in local keratinocytes, and these keratinocytes produced transgenic hTNF-α. Transgenic hTNF-α was observed to have a local biologic effect in the epidermis where it induced apoptosis of keratinocytes. Our results demonstrated that apoptosis of epidermal keratinocytes could be induced by TNF-α plasmid *in vivo*.

The molecular mechanisms of apoptosis are generally accepted to involve intracellular interactions induced by molecular signals. These signals, including Fas ligand, TNF-α, and other antigens, can interact with receptors, including Fas, TNF receptor, and T cell receptor, respectively. Messages from these receptors are transmitted to caspase. Some genes and proteins, such as c-myc, c-fos, Bcl-2, and p53 are also involved. Finally, endonuclease is activated and this leads to DNA fragmentation (Tartaglia *et al*, 1993; Hale *et al*, 1996; Tanuma, 1996). In this study, transgenic TNF-α was shown to bind TNF receptor in keratinocytes and induce apoptosis.

As a reliable method of *in vivo* gene transfer, plasmid DNA injection may be applied as keratinocyte gene therapy to treat certain skin diseases. In addition, this method can be useful for detection of biologic effects of certain genes in the skin *in vivo*. As for the possibility of clinical usefulness, keratinocyte gene therapy may be applied for some skin tumors by introducing TNF-α gene into tumor tissues by using the plasmid DNA injection method.

The authors would like to thank Ms. Y. Uno and K. Hanada for their excellent technical assistance.

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A Model for the Emergence of Café-au-Lait Macules

To the Editor:

Neurofibromatosis type 1 (NF1) or von Recklinghausen neurofibromatosis, is a disease with an incidence of about 1 in 3000 (von Deimling *et al*, 1995). This common heritable disorder is transmitted in an autosomal dominant form. Approximately 30%–50% of all cases do not have a family history of NF1 owing to new mutations of the NF1 gene (von Deimling *et al*, 1995). Diagnosis is mainly clinical and requires the following signs: café-au-lait spots, neurofibromas, glioma of the optic nerve, axillary lentigo, Lisch's nodules, and bone lesions (NIH Consensus Development Conference Statement, 1988; von Deimling *et al*, 1995). Café-au-lait spots are areas of hyperpigmentation of the skin consisting of an increased content of precursors of melanin macroglobules (Kaufmann *et al*, 1989; Eisenbarth *et al*, 1995). These benign lesions usually exhibit a smooth contour and are often the first symptoms of NF1. These possible manifestations make café-au-lait macules important for diagnosis (Ortonne *et al*, 1980; Huson *et al*, 1989). Seizinger (1993) suggested, based on the finding that a variety of unrelated tumor types are associated with NF1 gene aberrations, an importance of neurofibromin, the protein encoded by the NF1 gene, for the regulation of growth and the differentiation of a variety of cell types. The way neurofibromin acts may depend on cell type or developmental status (Seizinger, 1993; Griesser *et al*, 1997). Observed alterations in melanogenesis in cultured melanocytes from NF1 patients (Kaufmann *et al*, 1989; Kaufmann *et al*, 1991) are found to be related to a reduction of neurofibromin (Griesser *et al*, 1995). Griesser *et al* (1997) emphasize the importance of different neurofibromin levels after transcription, although no causal relationship between a reduced level of neurofibromin and an increase of café-au-lait macules has been described. The post-transcriptional regulation of neurofibromin levels may be caused by an unknown mechanism influencing both neurofibromin concentration and emergence of café-au-lait macules. In this study a model for the emergence of café-au-lait spots is proposed and supported by numerical simulations. The only assumptions that are made are the existence of the dependence of the increased melanin content on the concentration of an unknown substance and the existence of a critical level of this substance in a special phase of development.

How do these oval shaped spots emerge? Small differences in the initial concentration of the unknown postulated substance, which are responsible for the changed differentiation of melanocytes in café-au-lait areas, cause diffusion.

Assuming a critical level of concentration, at an early stage of development (before the appearance of café-au-lait spots but after diffusion), these pigmentation anomalies emerge naturally (see *Methods*). Simulating this model numerically we obtained the concentration landscape of **Fig 1**. Setting a threshold of a critical value of concentration – this amounts to cutting through the concentration landscape of **Fig 1** – we obtained the oval shaped spots of **Fig 1** (*lower panel*). Rarely, in these simulations irregular spots emerge. This is caused by an extremely uneven distribution of the initial concentration.

This study proposed a very simple model for the emergence of café-au-lait macules. It was inspired by the regulation of growth or survival of neurons during development by the nerve growth factor (Casaccia-Bonnefil *et al*, 1996; Davies *et al*, 1987). This model is based on differences in concentration that result in a diffusion process. These differences may be very small and caused by random deviations. We further assumed a link of this unknown substance, which is likely to be related to neurofibromin (Seizinger, 1993) but which does not concern neurofibromin, to the melanin content of melanocytes and a critical level of concentration needed for the emergence of these areas of hyperpigmentation. We do not postulate that the critical level of concentration needs to be below a certain value, it could be, but that it needs to be in a region, where it cuts a concentration landscape unevenly. Unlike another model (Riccardi, 1981), which relies on cell–cell interaction, the proposed model explains the form of café-au-lait spots with a diffusion process and a concentration threshold.

METHODS

We use a simple, analytic model of the skin, the two-dimensional euclidian space:

$$\mathbb{R}^2 = ((x, y): x \in \mathbb{R}, y \in \mathbb{R}).$$

We start with an arbitrary concentration of the unknown substance, $\rho_0(x,y)$. Then the concentration will equalize described by the simplest form of the diffusion equation:

$$\vec{j} = \tau \cdot \nabla\rho.$$

Where $\vec{j}$ is the flow of the substance, $\nabla\rho$ is the gradient of the concentration, and τ is the diffusion constant. The continuity equation

$$\mathrm{div}(\vec{j}) = \frac{\mathrm{d}}{\mathrm{d}t}\ \rho$$

leads to

$$\Delta\rho = \frac{1}{\tau}\ \frac{\mathrm{d}}{\mathrm{d}t}\ \rho.$$

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